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Assessment of Genome Integrity in Mitochondria of Testis Tissue, Cell Proliferation and Topoisomerase Assay in invitro Condition

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ABSTRACT

Aim: Assessment of genome integrity in testis and to explain the reasons for infertility with the drug metosartan.

Methodology: Comet assay with freshly isolated mitochondria, cryopreserved mitochondria and cryopreserved tissue mitochondria and cell proliferation assay by dye exclusion method and topoisomerase assay in fresh and deep freezed DNA.

Results and Discussion: Comet assay of isolated mitochondria both from fresh and cryopreserved samples was examined by fluorescence microscope, which revealed that cryopreservation of testes mitochondria samples lead to injury to the tissue and DNA fragmentation. Topo isomerase assay has showed that increased incubation leads to DNA fragmentation and cell proliferation assay with the drug has showed the 0% viability of the cells by trypan blue. So, in vivo studies are necessary to confirm whether the drug is safe or not.

Conclusion: Assessment of genome integrity lead to the finding that drug was not safe it resulted in so many difficulties in invitro condition.

Key Words: Mitochindria, Genome, Cryopreserved, Topoisomerase

INTRODUCTION

Comet assay is a combination of both agarose gel electrophoresis along with fluorescent microscope for detecting the DNA strand breaks in the nucleated cell. The extent of DNA migration is measure of damage in the genome of the cell. This is a simple technique as it doesn't require any radioactive labelling compounds and also problems associated with disposal. It can be more useful in case study of DNA damaging agents. The damaged DNA migrates as comet. The comet head consists of high molecular weight DNA and tail consists of the DNA single strand fragments. Applications of comet assay includes diverse things(1), in which change of lysis buffers and DNA stains resulted in different achievements like use of neutral buffer to find the double strand breaks whereas use of buffer containing specific endonucleases can used to find damage at base level. Here in my study I have taken neutral buffer to detect double stranded breaks in mitochondria isolated in pure form cryopreserved before the comet assay was performed. As the mitochondria is present in midpeice of sperm and responsible for motility of sperm , So studies on mitochondria genome is necessary as decreased motility of sperm leading to problems in fertility.

Cell proliferation assay is important to know the abnormal proliferation due to certain agents. Simple techniques like MTT assay and trypan blue assay is used to know whether the cells are normal or not (6). AS MTT assay of control gives the same result after the dye has entered in to the dead cell and So, useful to perform the trypan blue as the dye was taken by the non viable cells itself. In this study I have used metosartan to know the cell viability in vitro using trypan blue. The cells were dead and matched with the results of aniline blue staining.

Mammals express two types of topoisomerases alpha and beta which are homologous but differ in the sub cellular location of the enzyme. Similar to topoisomerase I and III,

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topoisomerase II also involved in relieving the topological constraints of DNA(2). Topoisomerase is homodimer binding to the clamps of DNA polymerase and the strand was cleaved by topoisomerase first using the ATP, the cleaved strand was passed through the gap, re ligated and the strands are released by hydrolysis of ATP after the release of ADP molecules.

In general sense DNA is unwinded in the replication in order to pass the genetic information or for transcription of the mRNA strands. In both the steps DNA winding is necessary but focussing on DNA replication there will be formation of positive supercoils and makes the the fork movement difficult an under winding of the DNA behind the fork also leads to torsional stress in the replication and leading to formation of catenated DNA . As we know helicases separates the two strands but not involved in the removal of torsional stress but here, the topoisomerases came in to role as that mentioned in the previous paragraph.

Topoisomerase II is also responsible for maintenance of chromatin integrity as supercoiled that means folded supra molecular structures both in fresh and frozen samples. The present aim is to know the genome integrity in the treated sample as in case of proliferation assay and fresh and frozen samples by topoisomerase assay and in fresh and cryopreserved samples in comet assay.

MATERIALS AND METHODS

Cell proliferation assay using trypan blue :(6)

1. The cell suspension was prepared in a balanced salt solution (e.g., Hanks' Balanced Salts [HBSS], and 0.5 ml of 0.4% Trypan Blue solution (w/v) was transferred to a test tube. To it 0.3 ml of HBSS is added along with 0.2 ml of the cell suspension (dilution factor = 5) and mixed thoroughly and Allowed to stand for 5 to 15 minutes after addition of metosartan drug to the suspension.
2. After proper incubation by using Pasteur pipette or any suitable device transfer the trypan blue- cell mixture suspension to both the chambers by just touching the tip of the pipette to the edge of the chamber and allowing it to fill by capillary action and overfilling or under filling was avoided.
3. Starting with the chamber 1 count all the four corners squares and repeat the procedure for chamber 2 also. Nonviable stains blue.

Cryopreservation of tissue

Liquid nitrogen was brought from S.V veterinary university and the tissue is homogenated in water and exposed to liquid nitrogen vapours and immersed in liquid nitrogen for 3 days and mitochondria was isolated after the cryopreservation and comet assay was performed. The isolated mitochondria was also cryopreserved, and processed in the same manner

Isolation of mitochondria from testes:(7)

After the sacrificing the animal, testes were removed, decapsulated and minced in isolation medium 250mM sucrose, 0.2 mM EGTA, 0.1mM EDTA,5mM HEPES-KOH (PH 7.4) and 0.1%defatted BSA. The tissue was homogenated in isolation medium and the homogenate was centrifuged at 10,000Xg for 10min and the supernatant was recentrifuged at 10,000Xg for 10min. The pellet was resuspended and repelleted twice in the isolation medium omitting EGTA, EDTA, & defatted BSA.

Comet assay: (3)

Standard comet assay was performed..100µl of the isolated mitochondria was mixed with 1000µl of 0.8% agarose and 1000µl of suspension was spread on the slide and kept in ice for 45min. After casting the slides was immersed in lysis buffer(2.5% SDS in 45 mM Tris–borate, 1 mM EDTA, pH 8.4) for 20min and kept in electrophoresis buffer for 5min. The electrophoresis was carried with TBE buffer at 2v/cm. Ethidium bromide staining was performed and visualised using fluorescent microscope.

Isolation of DNA from testis tissue :(4)

Cells were pelleted and the suspension was collected. To the supernatant lysis buffer (50mM tris-cl, PH8.0, 100MEDTA,0.125%SDS) was added and the solution was incubated in boiling water bath at 55^0c for overnight. To this 1ml of mixture of phenol :chloroform : isoamyl alcohol was added and centrifuged at 10,000rpm for 15min. Aqueous phase was collected and washed with 3ml of phenol: chloroform : isoamyl alcohol. Aqueous phase was collected and chilled ethanol was added and upper aqueous phase was separated and centrifuged at 10,000rpmfor 10min and the pellet was resuspended in 100µl of water.

Topoisomerase assay :(5)

The above isolated DNA was suspended in topoisomerase reaction buffer 10X (200mM tris-cl, PH:7.5, 100mM $Mgcl_2$, 10mMATP, 10mM EDTA, 10mM, β-mercaptoethanol, 1.5 M KCL,300µg/ml BSA. The suspension was incubated for 1-3hrs and agraose gel electrophoresis was performed with TAE buffer.

RESULTS

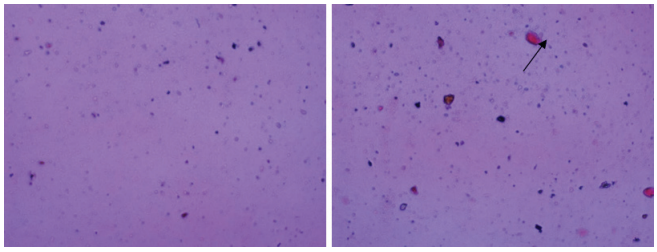

Figure 1: Single cell electrophoresis with fresh mitochondria isolated from testis sample. Mitochondria was isolated from the testis after sacrificing the animal and the resulted mitochondria was mixed with low melting agarose and 100µl of mixture was taken on the slide and smear was prepared and comet assay was performed. The comets observed are up to 1%.

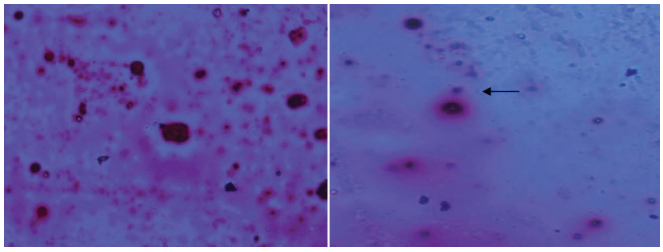

Figure 2: Single cell electrophoresis of isolated mitochondria from cryopreseved testis sample. Testis was isolated and chopped with scissors and cryopreserved by exposing to nitrogen vapours and plunged directly in to the liquid nitrogen. After cryopreservation mitochondria was isolated and comet assay was performed. The mitochondria have lost the integrity and micronuclei formation is seen.

Comet assay of fresh and cryopreserved testis sample mitochondria has given the conclusion that cryopreservation may result in distortion of shape, micronuclei formation which may result low motility of spermatozoa therefore leading to infertility. Incase of cryopreserved mitochondria after isolation showed less distortion and absence of micronuclei and also comets formed was similar to fresh sample.

In fresh sample the comets was up to acceptable extent than compared to the cryopreserved mitochondria testes samples.

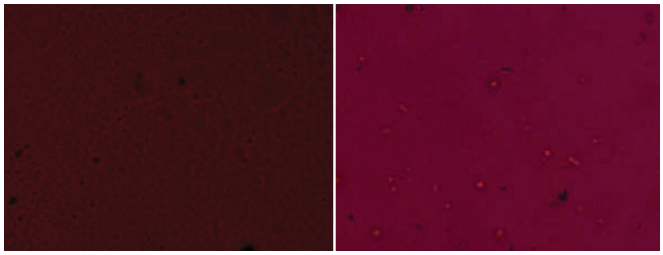

Figure 3: Single cell electrophoresis of cryopreserved mitochondria after isolation from testes sample. Mitochondria was isolated from testis and cryopreserved in liquid nitrogen and comet assay was performed with that mitochondria. The Comets formed are similar to that of fresh tissue .

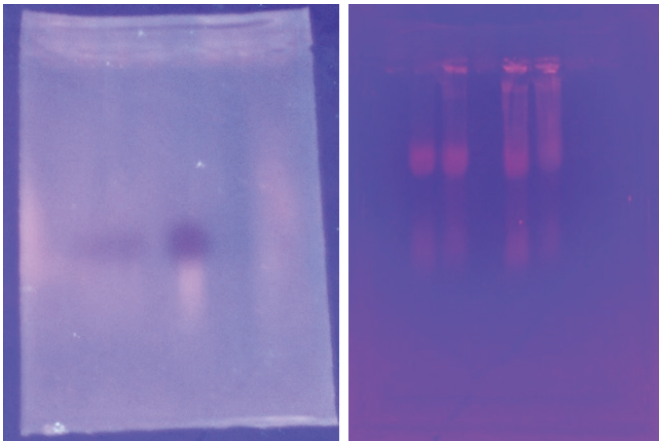

Figure 4: Figure: 4A describes the DNA isolated from testis and the purity was checked by agarose gel electrophoresis. Figure 4B describes the topoisomerase assay samples stored in refrigerator conditions. In first lane only DNA is present and in 2nd to 4th lane incubation of the DNA with topoisomerase enzyme and the extraction buffer and required reagents with 1-3hrs of incubation.

In the DNA isolated from the fresh tissue DNA fragments was not found where as in refrigerated samples treated with topoisomerase showed supercoiled structures and also the fragmented molecules which migrated to the longer distance in the gel. With increase in time period the intensity is also increased representing the cleavage.

Calculations:

Cell Counts – Each square of the haemocytometer, with cover-slip in place, represents a total volume of 0.1 mm^3 or 10-4 cm^3. Since 1 cm^3 is equivalent to approximately 1 ml, the subsequent cell concentration per ml (and the total number of cells) will be determined using the following calculations:

Cells Per mL = the average count per square × dilution factor × 10^4 (count 10 squares) If the average count per square is 15 cells × 4 × 10^4 = 6 × 10^5 cells/ml.

Total Cells = cells per ml × the original volume of fluid from which cell sample was removed.

6 × 10^5 (cells/ml) × 10 ml (original volume) = 6 × 10^6 total cells.

Cell Viability (%) = total viable cells (unstained) ÷ total cells (stained and unstained) × 100. If the average count per square of unstained (viable) cells is 0, the total viable cells = [0 × 4 × 10^4] viable cells/ml × 10 ml (original volume) = 0viable cells. Cell viability (%) = 0 (viable cells) ÷ 6 × 10^6 (total cells) × 100 = 0% viability.

From the cell proliferation assay it was clear that the drug incubation for 30min lead to 0% viability

DISCUSSION

Cell proliferation assay, topoisomerase assay and comet assay was used in the study for assessing the genome integrity. Comet assay was performed by so many people but for mitochondria is a new thing. Mitochondria isolated from the cryopreserved tissue showed micronuclei formation which indicates that DNA was fragmented and from fresh tissue revealed less by comets formation. Cell proliferation assay revealed that treatment with drug for 30min resulted in 0% viability. So, the drug was not safe and it should be confirmed with in vivo experiments also, in order to come to a conclusion.

CONCLUSION

The drug metosartan resulted in formation of micronuclei in mitochondrial genome and also decreased viability by trypan blue, topoisomerase assay has revealed that with incubation the DNA was fragmented resulting in the bands as supercoiled and fragmented DNA.

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